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Prevalence of cervical human papillomavirus is associated with p16 expression and irregular menstrual cycles among married Jordanian women

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Summary

Persistent infection with high-risk human papillomavirus (HPV) is the leading cause of cervical cancer worldwide. In this study, we used immunohistochemical methods to investigate the prevalence of HPV among Jordanian women who visited local gynaecological clinics. Expression of HPV and p16 protein was analysed to assess the high-risk prevalence of the virus. Accordingly, HPV was positively expressed in 41% (57 out of 140) of the cases and expression of p16 was found in 28% (39 out of 140) of the cases. Factor analysis showed that irregular cycles increased the chances of HPV by 13.342-fold ($p = 0.001$) and p16 expression by 17.091-fold ($p < 0.001$). Age-adjusted odds ratios were 12.923-fold for HPV ($p = 0.002$) and 16.986-fold for p16 expression ($p < 0.001$). In conclusion, awareness, screening, and vaccination are key preventive measures in the fight against HPV and cervical cancer are important in conservative countries such as Jordan.

Key words: Cervical human papillomavirus; p16; Menstrual cycle; Age-adjusted odds ratio.

Introduction

Cervical cancer is the second most common cancer in women worldwide. Almost all cases of cervical cancer are caused by persistent infection with one of nearly 15 genotypes of high-risk human papillomavirus (HPV) [1]. HPVs comprise a diverse group of viruses that display different epithelial tropisms and life-cycle strategies. A low-risk HPV group is highly prevalent yet rarely connected with neoplasia. By contrast, a high-risk HPV group has been associated with several human cancers including cervical cancer [2]. Early epidemiological studies on prevalence of HPV and cervical cancer predicted associations between geographical dissemination of different HPV strains and incidence of cervical cancer [3, 4]. Experimental evidence for biological mechanisms involved in HPV carcinogenesis are well established in literature [5]. Experiments show that species specific HPVs induce papillomas and cervical cancer in susceptible women. High-risk HPV oncogenes E6 and E7 trigger vital regulators of cell cycle progression, telomere maintenance, apoptosis, and chromosomal instability [6]. The p53 and retinoblastoma protein (Rb) are among the major targets of these oncogenes. E6 is one of the first genes expressed during HPV infection. This oncogene encodes a small 16–18 kD protein that initiates premature degradation of the p53 through a cellular ubiquitin-ligase, leading to bypass of normal growth arrest signals at the G1/S and G2/M cell cycle checkpoints [7-9]. On the other hand, the E7 oncogene encodes an 18 kD protein that binds and inactivates Rb protein and prevents it from binding to E2F which becomes free to stimulate cellular division and thereby promoting cell cycle progression [10]. These events lead to overexpression of p16 protein in high-risk HPV-infected cells. For this reason, p16 is now widely accepted as a sensitive and specific marker of squamous dysplastic cells of the cervix, and is a valuable adjunctive test in cervical cancer lesion diagnosis and cervical cancer screening [11]. The p16 is a cyclin dependent kinase inhibitor and a member of the INK4 family. It is coded by CDKN2 gene and produces a nuclear phosphoprotein with a molecular weight of 16 kD [12].

Genital HPV infection often goes unnoticed because the virus resides in the skin or mucous membranes and usually causes no symptoms. Some cases are presented with visible genital warts, or have pre-cancerous changes in the cervix, vulva, anus, or penis. Furthermore, HPV types that tend to cause genital warts are not the same ones that cause cervical cancer. Although, a wide variety of HPV types can cause genital warts, types 6 and 11 account for about 90% of all cases [13]. Genital warts usually appear as soft, moist, pink, or flesh-coloured swellings, usually in the genital

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Table 1. — *Clinical characteristics of 140 female subjects analysed.*

Characteristic	n=140
Age (mean ±SD)	37.6 ± 10.6
Age at marriage (mean ±SD)	20.7 ± 3.7
Months of marriage (mean ±SD)	16.9 ± 11.6
Number of pregnancies (mean ±SD)	4.3 ± 2.5
Discharge	
Normal	71
Abnormal	69
Menstrual cycle	
Regular	116
Irregular	15
Use of oral contraceptives	
Yes	11
No	129
Menopause	
Yes	9
No	131
Pap smear test	
Normal	134
Squamous cell carcinoma	4
ASCUS	2

area. After sexual contact with an infected person, warts may appear within weeks or months, or not at all [14]. Estimates predict that most sexually active men and women could acquire genital HPV infection at some point in their lives [15]. Sexual behaviour is a primary risk factor for infection. It has been found that the number of lifetime sexual partners is the most powerful predictor of HPV positivity in women tested for HPV [16]. Age is also an associated risk factor for HPV infection. In the population most at risk, those of reproductive age, as many as 75% may have been infected with genital HPV [17].

In Jordan, the occurrence of cervical intraepithelial neoplasia (CIN) is approximately 49 per 100,000 [18]. Genotyping of high-risk HPV in Jordan showed a prevalence of the virus in 87.2% of cervical cancer specimens with most predominant genotypes 16, 18, and 45 [19]. A previous report from Jordan also highlighted the prevalence of genotypes 16 and 18 in approximately 75% of invasive cervical cancer [20]. The study also showed the genotypes 39 and 56 to be more common than previously expected.

In this study, we investigated the prevalence of HPV among Jordanian women who visited local gynaecological clinics using immunohistochemical analysis. A broad-spectrum antibody was used to assess expression of HPV, while anti-p16 antibody was used to assess the high-risk prevalence of the virus.

Materials and Methods

A total of 157 Jordanian females examined at gynecologic clinics in King Abdulla University Hospital, Prince Hamza Hospital, Maternal and Child Health Centres, and Family Organization Centres, between the years 2007-2009, were included in this study. Two cervical smears from each participant were obtained according to standard procedures for HPV and p16 testing. Cells were scrubbed from the cervix using spatula, and then fixed on the slide using 96% ethanol. Participants were screened for cervical malignancies using Pap test at the same time of the study in local pathology laboratory. Cervical smear samples that were tested for HPV and p16 protein expression, were examined by a certified pathologist and the results were classified according to Klaes *et al.* scoring criteria [21] as described in the Immunostaining Assessment and Evaluation section.

Seventeen patient samples were excluded from the study because they were inadequate for evaluation. The clinical data for all the patients are summarized in Table 1.

This study was ethically approved by The Institutional Review Board (IRB) at Jordan University of Science and Technology (Irbid, Jordan).

Immunohistochemical detection of HPV was performed using commercially available HPV cocktail broad spectrum mouse monoclonal antibodies for (1, 6, 11, 16-16, 18, and 31) HPV types (clone BPV-1/1 H8+CAMVIR). Mouse anti-human p16 INK4a protein (clone E6H4) was also used for immunohistochemical determination of p16 protein overexpression in cervical smears.

Immunohistochemical detection of HPV and p16 was demonstrated using labelled sterptavidin biotin LSAB kit, which consists of secondary biotinylated goat anti-mouse antibody and conjugated sterptavidin horse radish peroxidase followed by DAB chromogene. Immunostaining was followed by counterstaining with hematotoxylin. Smears from cervical cancer patients known to express HPV and p16 protein were used as positive control. Smears incubated with antibody diluents instead of the primary antibody were used as negative control. All slides were examined under light microscope.

Heat-induced antigen retrieval for the cervical smears was carried out the reveal solution for 10 minutes. Subsequently, smears were washed and incubated with 1× PBS for five minutes. After that, endogenous peroxidise activity was blocked in the smear samples by incubation with 3% hydrogen peroxide for five minutes, followed by washing three times with PBS. Afterwards, smears were incubated for 30 minutes at room temperature with the chosen primary antibodies, and then washed three times with PBS. Negative control smears were not incubated with primary antibody. One drop of biotinylated secondary antibody was added to each smear and incubated for 15-20 minutes, then washed well with PBS. After that, they were incubated with sterptavidin peroxidase for 15-20 minutes at room temperature, and washed well with PBS. For visualization, smears were incubated with DAB chromogene for about five minutes, and washed with tab water. Smears were counterstained with hematoxylin for two minutes, then dehydrated through a series of alcohol (70%, 80%, 90%, and 100%). In the end, slides were mounted with DPX mounting medium and left to dry for examination under light microscope.

The immunohistochemical results were evaluated by Dr. Ali Shotar. A two-independent certified pathologist Scoring of HPV and p16 expression was performed following Klaes *et al.* scoring system [21], whereby the percentage of the cells, and reaction intensity were taken into consideration. For HPV, any cytoplasmic staining without nuclear staining was scored as negative, whereas nuclear and cytoplasmic staining was scored as positive for p16. The percentage was described for HPV, and p16 as follow: 0-10% (0), 10-30% (1), 30-85% (2), and > 85 % (3). The intensity of the reaction was scored as: negative (0), weak (1), moderate (2), and strong (3). The smears that were scored one and more were considered as positive.

To compare the three tests (Pap smear, HPV, and p16 expression) the Mann-Whitney test was used to test for clinical differ-

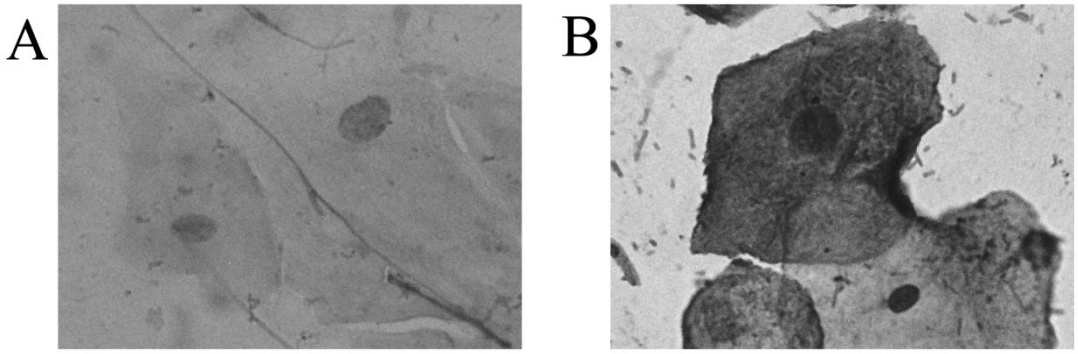

Figure 1. — HPV immunostaining of representative samples: (A) HPV negative cells and (B) HPV positive cells.

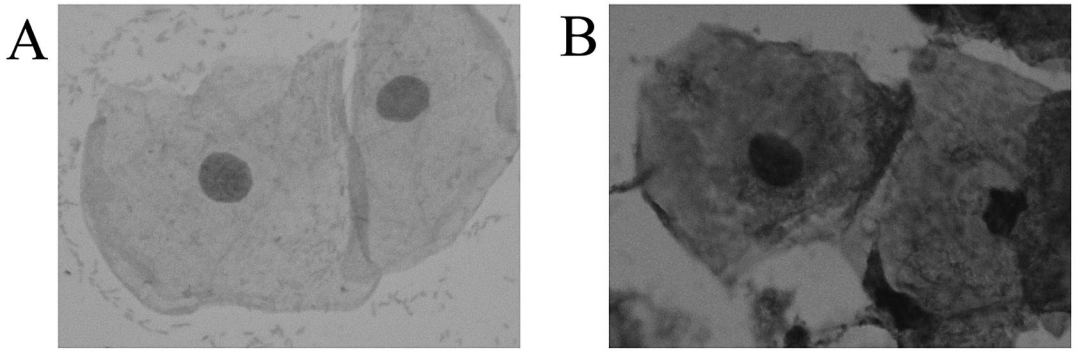

Figure 2. — p16 immunostaining of representative samples: (A) p16 negative cells and (B) p16 positive cells.

ences (age, age of marriage, and period of marriage) between patient groups. In addition, Fisher's Exact test was used to test for association between clinical characteristics (cycle regularity, menopause, discharge, and use of oral contraceptives). Binary logistic regression was used to measure odds ratios (OR). Univariate and age-adjusted OR were used to evaluate the role of clinical characteristics in the prevalence of HPV and p16 expression. A value of $p \leq 0.05$ was considered significant. The Statistical Package for the Social Sciences program was used for data analysis (SPSS 16.0).

Results

A total number of 157 Jordanian females who were examined by local gynaecologic clinics were included in this study (Table 1). Seventeen patient samples were excluded from the study because they were inadequate for evaluation. The participant's median age was 36 (range 21 to 70) years, while the median age of marriage was 20 (range 14 to 29) years. The median for months of marriage was 14 (range 1 to 56). The median of the number of pregnancies was four (range 0 to 13). Out of the 140 participants, 69 participants were suffering from abnormal discharge and 15 participants had irregular cycle, while nine were in menopause. Eleven participants had used oral contraceptives as a tool for birth control.

Abnormal Pap test results were reported in 4% (six out of 140) of the cases. Two patients out of the six were diagnosed with the presence of atypical squamous cells of undetermined significance (ASCUS) and the remaining four cases were diagnosed to have squamous cell carcinoma.

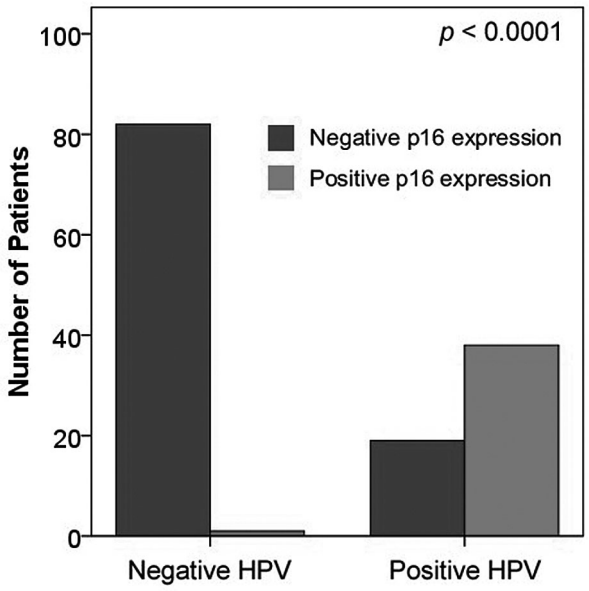

Figure 3. — Relationship between HPV and p16 expression.

Positive expression of HPV was found in 41% (57 out of 140) of the cases, and overexpression of p16 was found in 28% (39 out of 140) of the cases. Representative results of

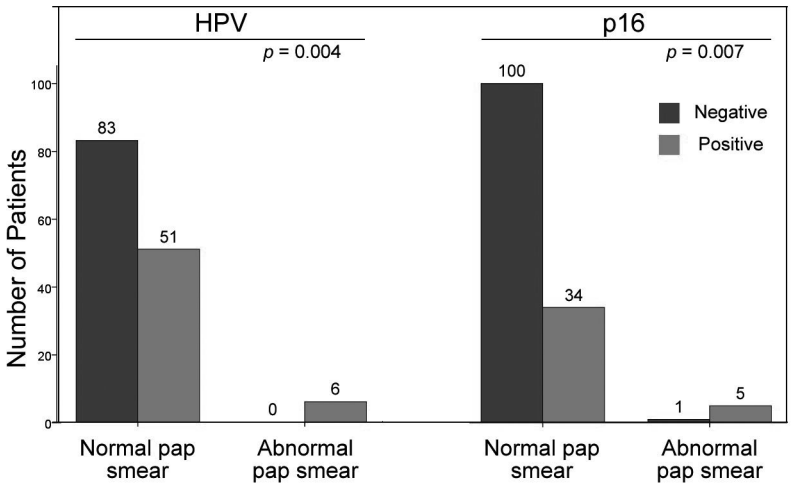

Figure 4. — Relationship between Pap smear, HPV, and p16 expression.

immunohistochemical staining of HPV and p16 are shown in Figures 1 and Figure 2, respectively. The results clearly indicate a statistically significant association between positive HPV results and the p16 overexpression ($p < 0.0001$) (Figure 3).

As mentioned earlier, there was a statistically significant association between p16 overexpression and the presence of HPV infection. Furthermore, a statistically significant association between abnormal Pap smear and the positive expression of HPV and p16 was also reported in our data (Figure 4). All the six abnormal Pap smear cases were reported to be in premenopause. Five out of the six abnormal Pap smear cases presented with irregular cycles. Four abnormal Pap smear cases presented with abnormal discharges. Only one abnormal Pap smear case was reported to be using oral contraceptives. Despite the low number of abnormal cases, there was a statistically significant association between the abnormal Pap smear results and cycle irregularity ($p < 0.0001$). Also, cases of abnormal Pap smear show a statistically significant relation with older age, but not with age of marriage or period of marriage ($p = 0.03$).

For the HPV test results, it was reported that at the time of the study, 51 out of 57 positive HPV cases were premenopausal, and 13 out of 51 positive HPV cases presented with irregular cycle (cases at menopause were excluded). Twenty-five out of 57 positive HPV cases presented with abnormal discharges.

Only five cases out of 57 positive HPV cases were reported to have been using oral contraceptives at some time in their lives. Positive HPV cases were significantly associated with experiencing irregular cycle ($p < 0.0001$). Also, cases of positive HPV show a statistically significant relation with older age, but not with age of marriage or period of marriage ($p = 0.03$).

The results of p16 test showed that 34 out of 39 positive p16 cases were premenopausal. Twelve out of 34 positive p16 cases presented with irregular cycles (cases at menopause were excluded). Eighteen out of 39 positive p16 cases presented with abnormal discharges, and only three cases of positive p16 were reported using oral contraceptives. Positive p16 cases show a statistical significant relation with irregular cycle ($p < 0.0001$). Also, cases of positive p16 expression show a statistically significant relation with older age, but not with age of marriage or period of marriage ($p = 0.006$).

To evaluate the role of clinical characteristics in the prevalence of HPV and p16 expression, the authors calculated univariate OR (Table 2) and age-adjusted OR (Table 3). Age, months of marriage, and irregular cycles were significant factors that increased the chances of HPV and p16 expression as shown in univariate analysis (Table 2). Most notably, irregular cycles increased the chances of HPV by 13.342-fold ($p = 0.001$) and p16 expression by 17.091-fold ($p < 0.001$) as shown in Table 2. The age of marriage, number of pregnancies, menopause, abnormal discharge or use of oral contraceptives did not show any significant effect on OR.

OR adjusted to age were used to evaluate the role of these factors, while taking into consideration the significant role of age. The role of months of marriage was apparently a result of age, as the statistical significance was lost in this test (Table 3). Age-adjusted OR for cycle irregularity remained statistically significant, indicating that this factor is independent of age. When age was added to the regression equation, women with cycle irregularity had 12.923-fold more chances of positive HPV ($p = 0.002$) and 16.986-fold more chances of positive p16 expression ($p < 0.001$). Like univariate binary logistic regression, age-adjusted OR did not show significant effect for the age of marriage, number of pregnancies, menopause, abnormal discharge or use of oral contraceptives.

Since cycle irregularity is often associated with hormonal

Table 2. — *Risk factor analysis by univariate binary logistic regression.*

Characteristic	Group	HPV			p16		
		OR	CI 95%	*p*-value	OR	CI 95%	*p*-value
Age		1.044	1.010-1.080	0.012	1.054	1.017-1.093	0.004
Age at marriage		1.031	0.942-1.130	0.507	1.014	0.918-1.121	0.778
Months of marriage		1.033	1.002-1.064	0.035	1.042	1.010-1.076	0.01
No. of pregnancies		0.987	0.860-1.133	0.852	0.988	0.849-1.150	0.876
Menopause	Pre-	1			1		
	Post-	3.137	0.751-13.106	0.117	3.566	0.905-14.056	0.069
Cycle	Regular	1			1		
	Irregular	13.342	2.865-62.134	0.001	17.091	4.441-65.770	<0.001
Discharge	Normal	1			1		
	Abnormal	0.692	0.352-1.364	0.288	0.84	0.401-1.762	0.645
Oral contraceptives	No	1			1		
	Yes	1.234	0.358-4.255	0.739	0.969	0.243-3.857	0.964

OR: odds ratio. CI 95%: confidence interval.

Table 3. — *Risk factor analysis by age-adjusted binary logistic regression.*

Characteristic	Group	HPV			p16		
		OR	CI 95%	*p*-value	OR	CI 95%	*p*-value
Age at marriage		1.049	0.955-1.152	0.322	1.041	0.940-1.154	0.438
Months of marriage		0.954	0.868-1.048	0.322	0.96	0.867-1.064	0.438
No. of Pregnancies		0.851	0.715-1.013	0.069	0.829	0.684-1.005	0.057
Menopause	Pre-	1			1		
	Post-	1.018	0.168-6.167	0.985	0.854	0.141-5.179	0.863
Cycle	Regular	1			1		
	Irregular	12.923	2.499-66.839	0.002	16.986	3.765-76.636	<0.001
Discharge	Normal	1				1	
	Abnormal	0.843	0.415-1.712	0.636	1.129	0.511-2.497	0.764
Oral contraceptives	No	1			1		
	Yes	1.244	0.354-4.369	0.733	0.992	0.243-4.044	0.991

OR: odds ratio. CI 95%: confidence interval.

imbalance, the authors further investigated the possibility of bias in their data. Fisher exact test was used to check for bias in analysis that can be caused by dependency of cycle regularity on contraceptives. No relationship was found between cycle and use of contraceptives ($p = 0.6$). Only one woman out of 15 that reported irregular cycle had used oral contraceptives. While for the 116 women reporting regular cycles, ten have used oral contraceptives. The remaining nine women were not applicable for cycle regularity and all of them were in the category that did not use contraceptives.

Discussion

Cervical cancer is the second most common cancer in women worldwide, arising from persistent infection of high-risk HPV virus [1]. Preventive measures have been employed worldwide to fight cervical cancer. These include screening of women aged 25 and above for early detection [22, 23] and vaccination, cytological abnormalities, and lesions associated with HPV [24]. Two vaccines have proved to be highly effective in preventing HPV infection from corresponding species of HPV: Gardasil a quadrivalent vaccine against HPV types 6, 11, 16 and 18 and Cervarix which is a bivalent vaccine against HPV types 16 and 18 [25].

In Jordan, the awareness and knowledge about HPV risks and prevention are progressing amongst university female students [26], obstetricians, and gynaecologists [27]. Survey among female students showed that most of the respondents (68%) had knowledge of the virus transmission and 59% of them knew that persistent infection with HPV virus can cause cancer. Only 45% of the students had never heard about HPV vaccine and 38% of them knew about its effectiveness [26]. This contrasts with nearly 80% of professionals who knew about the vaccine and its effectiveness [27].

While the prevalence of HPV genotypes in Jordan is similar to those in Western countries [19, 20], Jordan is considered conservative regarding sexual relationships. Indeed, previous report by Dajani *et al*., showed that marital status and circumcision in male partners were significant determinants of cervical cancer in Jordan [18]. Furthermore, a comparison with non-Jordanian females residing in Jordan, at the time of study, highlighted the role of cultural factors in determination of risk factors, including: age at marriage

and average duration, parity, breast feeding, oral contraceptives, socioeconomic status, and menstrual disorders. The study recommended the use of Pap smears in females over 20 years of age.

The role of clinical presentation in the prevalence of HPV and p16 expression was evident in the present study. Age, months of marriage, and irregular cycles were significant factors that increased the chances of HPV and p16 expression as shown in univariate OR analysis. Most notably, irregular cycles increased the chances of HPV by 13.342-fold ($p = 0.001$) and p16 expression by 17.091-fold ($p < 0.001$). The age of marriage, number of pregnancies, menopause, abnormal discharge or use of oral contraceptives did not show any significant effect on OR. The present authors used age-adjusted OR to evaluate role of these risk factors, while taking into consideration the significant role of age. The role of months of marriage was apparently a result of age, as the statistical significance was lost in this test. Age-adjusted OR for cycle irregularity remained statistically significant, indicating that this factor is independent of age. When age was added to the regression equation, women with cycle irregularity had 12.923-fold more chances of positive HPV ($p = 0.002$) and 16.986-fold more chances of positive p16 expression ($p < 0.001$). Like univariate OR, age-adjusted OR did not show a significant effect on the age of marriage, number of pregnancies, menopause, abnormal discharge or on the use of oral contraceptives.

Conclusions

Preventive measures in the fight against HPV and cervical cancer are important in conservative countries like Jordan. Awareness, screening, and vaccination have been proved as effective preventive methods in various countries around the world. However, both genetic and cultural differences can influence the risk factors that play a role in virus dissemination. Jordan hosted and still hosts the major human emigration events in the Middle East in the 20th and 21st centuries, leading to massive genetic and cultural changes. Research towards identification of risk factors and rapid changes in viral prevalence are important in establishing effective preventive measures for cervical cancer.

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